EFFECT OF PREP-MODIFICATIONS ON AUTOPHAGY

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Tiivistelmä/Referat – Abstract

Autofagian avulla solut hajottavat solunsisäisiä komponentteja, joita ei enää tarvita tai jotka ovat haitallisia soluille. Se on tärkeää solujen homeostaasin ja selviytymisen kannalta, ja autofagia on yhdistetty useisiin sairauksiin ja patofysiologiaan. Autofagia on monimutkainen prosessi, johon liittyy useita epäselviä ja tuntemattomia näkökohtia. Autofagian säätely on olennaista epätoivotun ja liiallisen aktivaation välttämiseksi ja siihen osallistuu useita signaalinvälitysreittejä ja molekyylejä, sekä aktivoivia että estäviä. Eri säätelyreitit ovat herkkiä erilaisille ympäristön signaaleille. Kaksi tärkeintä autofagiareittiä ovat mTOR-riippuvainen ja -riippumaton säätelyreitti. Jälkimmäisessä säätelyreitissä autofagian induktio riippuu Bcl-2:n ja Beclin 1:n vuorovaikutuksesta. Prolyylioligopeptidaasi (PREP) on peptidaasientsyymi, jolla on useita substraatteja. Sen inhibitio KYP-2047-molekyylillä voi vähentää α-synukleiinin aggregaatiota joko lisäämällä autofagiaa tai vähentämällä dimerisaatiota. Tämän työn tarkoitus oli selvittää miten PREP vaikuttaa Bcl-2:n ja Beclin 1:n väliseen vuorovaikutukseen ja mitä vaikutuksia sillä on autofagiaan. Edellisten tutkimusten perusteella, PREP-inhibitio näyttäisi lisäävän Beclin 1:n määrää ja vaikuttaa Bcl-2:n ja Beclin 1:n fosforylaatioon ja siten niiden muodostaman kompleksin hajoamiseen. Hypoteesina oli löytää eroja Bcl-2:n ja Beclin 1:n kolokalisaatiossa eri PREP-modifikaatioilla käsitellyissä soluissa ja oletettiin PREP-inhibition lisäävän kolokalisaation määrää. Kokeissa käytettiin HEK-293-soluja (human embryonic kidney cells 293) ja niistä CRISPR/Cas9-menetelmän avulla tehtyä hPREP poistogeenistä solulinjaa. Tavallisilla HEK-soluilla tehtiin kaksi koetta: inhibiittorikoe KYP-2047-molekyylillä (1 tai 10 µM) ja yliekspressiokoe (transfektio joko aktiivisella tai inaktiivisella hPREP-plasmidilla). Soluille tehtiin immunofluoresenssivärjäys, jonka jälkeen ne kuvattiin konfokaalimikroskoopilla ja tehtiin Bcl-2:n ja Beclin 1:n kolokalisaatioanalyysi. Beclin 1:n intensiteetti tumissa oli voimakkaampi kuin solun muissa osissa kaikissa näytteissä. Tämä voi viitata esim. siihen, että sen tumansisäiset tehtävät ovat autofagiaa aktiivisempia. Todennäköisesti kuitenkin immunofluoresenssivärjäyksessä käytetty vasta-aine on aiheuttanut havaitun värjäytymismallin. Edellisten tutkimusten perusteella odotettiin, että eri PREP-modifikaatioilla käsiteltyjen solujen välillä on eroa Bcl-2:n ja Beclin 1:n kolokalisaatiossa. Merkittäviä eroja kolokalisaatiossa ei kuitenkaan havaittu missään koeasetelmassa vaan kolokalisaatio oli lähes 100 prosenttia kaikissa näytteissä. Autofagian määrää soluissa ei mitattu, joten on mahdoton määrittää, oliko autofagian määrässä muutoksia, jotka eivät ole näkyneet muutoksina kolokalisaatiossa. On mahdollista, että jopa pieni muutos kolokalisaatiossa vaikuttaa autofagian määrään tai Bcl-2- ja Beclin 1-proteiineilla voi olla alapopulaatioita, joissa niiden välinen vuorovaikutus on häiriintynyt ja nämä muutokset ovat niin pieniä, ettei niitä voi havaita tässä tutkimuksessa käytetyillä menetelmillä. Molemmilla proteiineilla on soluissa myös autofagiaan liittymättömiä tehtäviä, jotka saattavat vaikuttaa tässä tutkimuksessa saatuihin tuloksiin.

Avainsanat – Nyckelord – Keywords
Autofagia, prolyylioligopeptidaasi, Bcl-2, Beclin 1, HEK-293, KYP-2047, immunofluoresenssivärjäys, konfokaalimikroskopia, kolokalisaatioanalyysi

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Tiivistelmä/Referat – Abstract

Autophagy is a pathway for cells to degrade intracellular components that are no longer needed or are detrimental for the cells. It is essential for cell homeostasis and survival and has been related to various diseases and pathophysiology. Autophagy is a complex process and there are still several unclear und unknown aspects to it. Regulation of autophagy is essential to prevent unwanted and escess activation, and several pathways and molecules, both stimulatory and inhibitory, are included. Different signaling pathways are sensitive to a variety of environmental clues. Two main autophagy pathways are mTOR-dependent pathway and mTOR-independent pathway. Induction of autophagy in the latter pathway is dependent on the interaction of Bcl-2 and Beclin 1. Prolyl oligopeptidase (PREP) is a peptidase enzyme that has several substrates. PREP-inhibition by KYP-2047 can reduce aggregation of α-synuclein in two ways: by increasing rate of autophagy and by decreasing dimerization. The aim of this study was to find out how PREP affects the interaction between Bcl-2 and Beclin 1 and how this affects autophagy. Based on previous studies, PREP-inhibition seems to increase the amount of Beclin 1 and to affect the phosphorylation of Bcl-2 and Beclin 1, leading to dissociation of the complex. Hypothesis was to see differences in colocalization of Bcl-2 and Beclin 1 in cells treated with different PREP-modifications and for PREP-inhibition to decrease the colocalization. Human embryonic kidney cells 293 (HEK-293) and hPREP knockout cell line created from them by using CRISPR/Cas9-silencing were used in the experiments. Two experiments were performed on regular HEK-cells: inhibitor experiment with KYP-2047 (1 or 10 µM) and overexpression experiment (transfection with either active or inactive hPREP plasmid). After immunofluorescence staining, cells were analysed with confocal microscope and colocation analysis of Bcl-2 and Beclin 1 was performed. The intensity of Beclin 1 in the nuclei was stronger than in other parts of the cell in all samples, which could indicate a stronger activity of its nuclear tasks compared to autophagy. However, the antibody used for immunofluorescence has most likely caused this staining pattern. Based on previous knowledge, it was expected to see differences in colocalization of Bcl-2 and Beclin 1 in cells treated with different PREP-modifications. However, there were no significant differences in colocalization of Beclin 1 and Bcl-2 in any of the experiments but it was nearly 100 percent in all treatments. Since rate of autophagy in cells was not detected, it is impossible to determine, if there were changes in autophagy that were not reflected as changes in colocalization of these two proteins. It is possible that even a small change in colocalization can affect the rate of autophagy or there might be subpopulations where the interaction is interrupted and these changes are so small that they are not detectable with the methods used in this experiment. Both Bcl-2 and Beclin 1 also have functions not related to autophagy, which could be one reason behind the results gained in this study.

Avainsanat – Nyckelord – Keywords
Autophagy, prolyl oligopeptidase, Bcl-2, Beclin 1, HEK-293, KYP-2047, immunofluorescence staining, confocal microscopy, colocation analysis

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CONTENTS

1 AUTOPHAGY IN MAMMALIAN CELLS

Macroautophagy is a pathway for cells to degrade intracellular components that are no longer needed or are detrimental for the cells (Yang and Klionsky 2010). It is essential for cell homeostasis and survival and has been related to various diseases and pathophysiology. It is also an adaptive process to reply to energy-demand of the cell in both normal conditions and under environmental stress. In addition to macroautophagy, there are also other types of autophagy in mammalian cells that take place at lysosome membrane (Xie and Klionsky 2007). In microautophagy, cytoplasmic content is directly engulfed by lysosome whereas in chaperone-mediated autophagy unfolded proteins are translocated across lysosome membrane. Macroautophagy (hereafter autophagy), uses specialised vesicles called autophagosomes, that fuse with lysosomes to degrade cytoplasmic material (Yang and Klionsky 2010). Autophagy is a complex process and there are still several unclear und unknown aspects to it. Autophagy can be both beneficial and harmful to cells as it is a protective mechanism, but can also lead to unnecessary cell death. Regulation of autophagy is essential to prevent unwanted activation, and several pathways and molecules are involved. Crosstalk with apoptosis might also be important in some developmental respects and disease pathogenesis.

1.1 Formation of autophagosomes

Central process in autophagy is the formation of autophagosomes that deliver the material to lysosomes where degradation takes place (Xie and Klionsky 2007). Formation takes place in specialised regions of the endoplasmic reticulum (ER). Autophagy starts when phagophore sequesters cytoplasmic material and double-membrane vesicle, autophagosome forms (Figure 1). Autophagosome outer membrane then fuses with lysosome that contains hydrolases and autolysosome forms. When exposed to enzymes, the inner compartment and its contents are degraded and degradation products recycled. Formation of autophagosome is a complex process that is regulated by autophagy-related genes (*ATG*), some of which are conserved through evolution and have homologues in different organisms from yeasts to mammals.

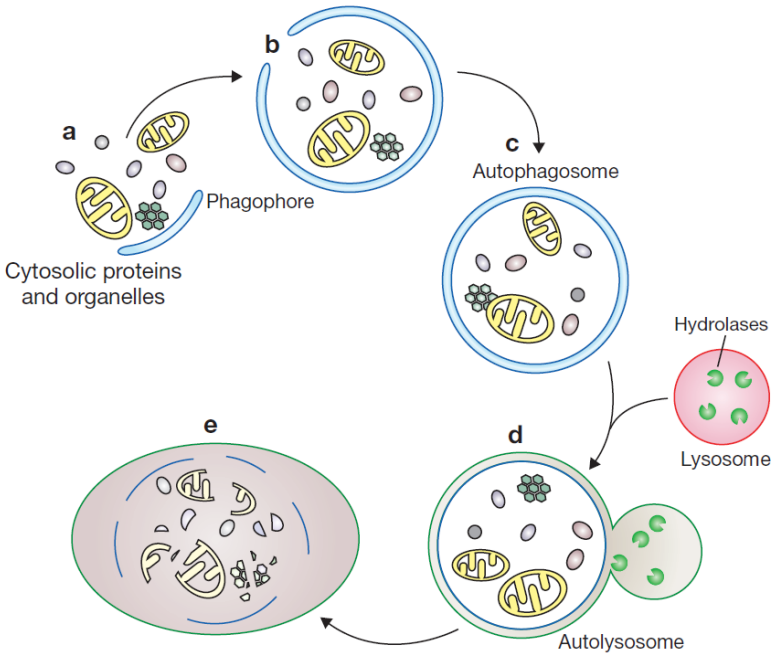

Figure 1. Formation of autophagosome and fusion with lysosome. Phagophore engulfs cytosolic material (a, b) and autophagosome forms (c). Outer membrane of autophagosome fuses with lysosome and inner membrane is exposed to lysosomal enzymes (d), which leads to degradation of cytosolic material (e). (Xie and Klionsky 2007)

In mammals, initiation of autophagy and formation of phagophore requires ULK1/2 complex, that can be inactivated by mTORC1 (mammalian target of rapamycin complex 1) (Yang and Klionsky 2010). Class III phosphatidylinositol 3-kinase (class III PtdIns3K) complex 1, which consists of Vps34 (vacuolar protein sorting 34), Vps15, Beclin 1 (Atg6), and Atg14, is essential in autophagosome formation. Origin of autophagosome membrane is still unclear (Weidberg et al 2011) but mammalian Atg9 (mAtg9) is potentially involved in delivery of the membrane (Yang and Klionsky 2010). LC3-II and Atg12-Atg5-Atg16L complex are conjugation systems that most likely have a role in elongation of phagophore membrane as the autophagosome forms. Microtubule network is probably an important contributor in autophagosome formation and trafficking and might even be involved in autophagosome maturation (Weidberg et al 2011).

Autophagy was previously seen as nonselective, except for some selective forms of autophagy, but later research has provided evidence that autophagy is a selective process (Weidberg et al 2011). Cargo selection for autophagosomes is mediated in several different ways depending on

the type of cargo. For example, cytosolic protein clearance is mediated by autophagic adaptors like p62 protein (p62/SQSTM1).

1.2 Autophagy pathways

Regulation of autophagy is a complex network that consists of several stimulatory and inhibitory factors. Different signaling pathways are sensitive to a variety of environmental clues like amino acids, growth factors and glucose and ATP-levels (Rubinsztein et al 2012). There are two major signaling pathways in autophagy. Other pathway involves mTOR and is important especially in amino acid deprivation induced autophagy (Jung et al 2010). The other main autophagy pathway is mTOR-independent.

1.2.1 mTOR-dependent autophagy

TOR is a serine/threonine protein kinase and the target protein of rapamycin (Jung et al 2010). It is conserved through evolution in yeast and mammalian cells. mTOR has several functions in cells and a key molecule in signaling network that regulates cell growth and autophagy pathways. mTOR integrates class I PtdIns3K signalling and amino acid-dependent signalling. Active mTOR activates protein synthesis and other pathways that are essential for cell growth and inhibits autophagy. With different binding partners, mTOR forms two complex proteins called mTORC1 and mTORC2 that have different functions in the cells. mTORC1 integrates several pathways regulating autophagy activity in response to nutritional status (nutrient and growth factor levels) and cellular stress (Figure 2). These upstream signaling pathways are complex and several details remain unknown. mTORC2, on the other hand, regulates autophagy in skeletal muscle through a separate pathway (Mammucari et al 2007).

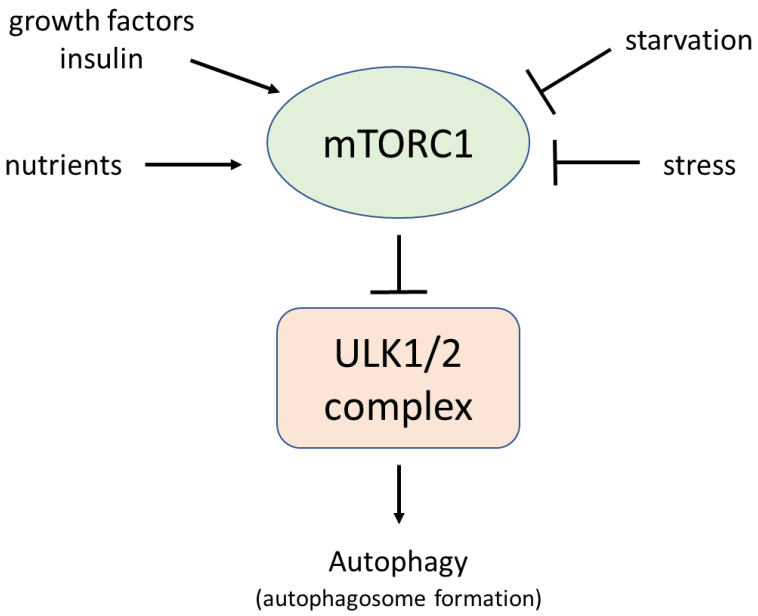

Figure 2. mTORC1 is regulated by nutrient and growth factor/insulin levels that promote its activation. Starvation and stress conditions inhibit mTORC1 and make activation of ULK1/2 complex possible which leads to autophagy and formation of autophagosomes. Adapted from Jung et al 2010.

Downstream of mTORC1, ULK1/2 complex (Figure 2) is an important regulator of autophagy and autophagosome formation and is inactivated by mTORC1 (Yang and Klionsky 2010). Complex consists of ULK1/2 and its binding partners (Jung et al 2010). Mammalian homologue of Atg13 interacts with ULK1/2 (Jung et al 2009). mTORC1 can phosphorylate Atg13 and also ULK1/2 in vitro and inhibition by mTORC1 leads to dephosphorylation of complex (Ganley et al 2009, Jung et al 2009). mTORC1 reduces kinase activity of ULK1/2, which could be through altering cellular location of ULK. Exact phosphorylation sites in ULK complex have not been determined (Jung et al 2010). ULK C-terminal residues regulate both kinase activity of ULK and its autophagosomal location (Chan et al 2009). C-terminal region has been suggested to mediate ULK binding proteins and to regulate ULK conformation.

1.2.2 mTOR-independent autophagy

The mTOR-independent pathway is regulated by JNK1 (c-Jun N-terminal kinase 1) that phosphorylates Bcl-2 (B-cell lymphoma 2), and DAPK (death-associated protein kinase) and AMPK (AMP-activated protein kinase) (Zhang et al 2016) that phosphorylate Beclin 1 (Figure 3)

(Yang and Klionsky 2010). Interaction between Bcl-2 and Beclin 1 involves a BH3 domain located in Beclin 1 (Maiuri et al 2007). Phosphorylation of either Bcl-2 or Beclin 1 interferes with their association, which leads to activation of class III PtdIns3K complex associated with Beclin 1 and induction of autophagy (Yang and Klionsky 2010). Interaction can also be regulated by competitive binding of BIK (Bcl-2 interacting killer) with Bcl-2 (Chang et al 2010). Beclin 1 and Bcl-2 have some differences in their localization in cells. Endogenous Beclin 1 localizes to trans-golgi network (TGN) (Kihara et al 2001), but can also colocalize with mitochondria and ER (Pattingre et al 2005) whereas Bcl-2 localizes on outer ER and mitochondrial membranes, but not to TGN (Germain and Shore 2003).

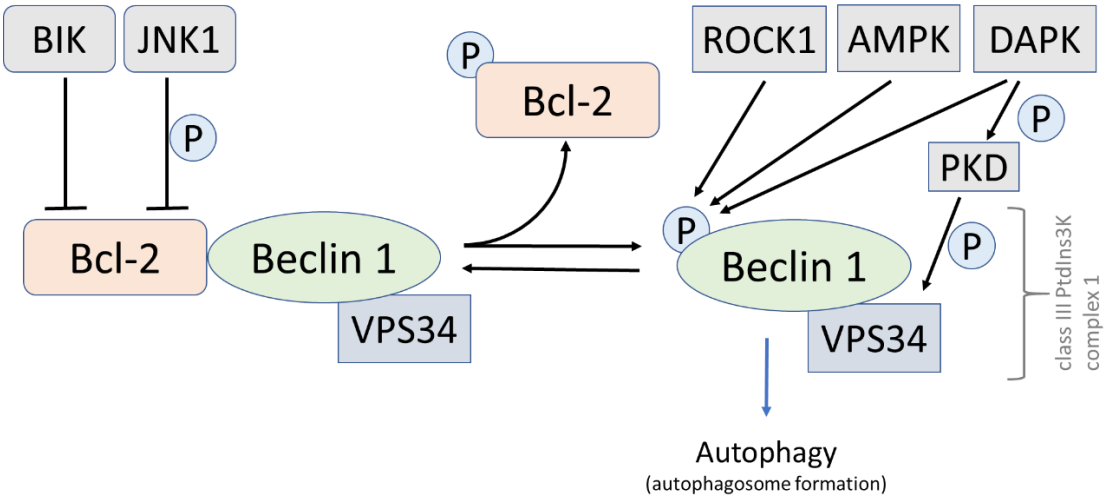

Figure 3. Regulation of interaction between Bcl-2 and Beclin 1. Binding of Bcl-2 to Beclin 1 can be regulated by competitive binding with BIK or phosphorylation by JNK1. Beclin 1 can be phosphorylated by different kinases (ROCK1, AMPK and DAPK). Dissociation of Bcl-2 and Beclin 1 leads to autophagy.

Since phosphorylation and interaction of Beclin 1 and Bcl-2 is crucial for their autophagic activity, it should be carefully controlled. DAPK is calcium/calmodulin regulated serine/threonine kinase that has several tasks in cells in addition to regulating autophagy (Levin-Salomon et al 2014). DAPK regulates autophagy by modulating class III PtdIns3K complex by two mechanisms. It can activate a kinase cascade and phosphorylate protein kinase D (PKD) which then activates Vps34 (part of PtdIns3K complex). Other option is that DAPK directly phosphorylates Beclin 1 on Thr119 (Zalckvar et al 2009). This same location on Beclin 1 can also be phosphorylated by ROCK1 during starvation-induced autophagy (Gurkar et al 2013). DAPK

might also affect autophagy by modulating microtubule dynamics (Levin-Salomon et al 2014). Microtubules are required in phagophore formation and trafficking of autophagosomes to lysosomes and thus an intact microtubule cytoskeleton is essential for autophagy. Starvation conditions induce multisite phosphorylation on Bcl-2 mediated by JNK1 (Wei et al 2008). JNK1 phosphorylates Bcl-2 at Thr69, Ser70 and Ser87.

1.3 Autophagy in neurodegenerative diseases

Autophagy is altered in several diseases, including cancer, and in immunity and aging (Yang and Klionsky 2010). Autophagy is also important in neurodegenerative diseases and to maintain homeostasis in neural cells. Loss of *Atg7*, an essential gene for autophagy, has been shown to cause neurodegeneration in mice (Komatsu et al 2006). Autophagy affects degradation of several aggregate-prone proteins that are involved in disease pathogenesis, such as α-synuclein (Yang and Klionsky 2010). Autophagy-inducer rapamycin has been shown to enhance clearance of different aggregate-prone proteins and to reduce their toxicity such as huntingtin and tau, which are key proteins in Huntington's disease and Alzheimer's disease, respectively (Berger et al 2006). Chaperone-mediated autophagy and macroautophagy can both degrade wild type α-synuclein in cells (Webb et al 2003, Xilouri et al 2008). Disturbances in autophagy thus affect accumulation of α-synuclein and pathogenesis of Parkinson's disease. On the other hand, overexpression of α-synuclein inhibits autophagy by affecting autophagosome formation (Winslow et al 2010). Beclin 1 co-expression in α-synuclein-overexpressing cells has been shown to activate autophagy and reduce α-synuclein accumulation in cells (Spencer et al 2009).

2 AUTOPHAGY AND PREP

Prolyl oligopeptidase (PREP) is a peptidase enzyme that has several substrates (Garcia-Horsman et al 2007). PREP can increase aggregation of α-synuclein and PREP-inhibition by KYP-2047 (4-phenylbutanoyl-L-prolyl-2(S)-cyanopyrrolidine) enhances clearance of protein aggregates by increased rate of autophagy (Savolainen et al 2014). PREP seems to reduce formation of autophagosomes but its inhibition increases expression of Beclin 1, leading to enhanced autophagy. PREP has been shown to interact directly with α-synuclein and this binding of PREP with α-synuclein increases aggregation (Savolainen et al 2015). PREP-inhibition can thus reduce

aggregation of α-synuclein in two ways: by increasing rate of autophagy and by decreasing dimerization.

3 AIMS AND HYPOTHESES

The aim of this study was to find out how PREP affects the interaction between Bcl-2 and Beclin 1 and how this affects autophagy. Based on previous studies, PREP-inhibition seems to increase the amount of Beclin 1 and to affect the phosphorylation of Bcl-2 and Beclin 1, leading to dissociation of the complex (Svarcbahs, Julku, Jakola, Savolainen, Myöhänen, manuscript 2017). Hypothesis was to see differences in colocalization of Bcl-2 and Beclin 1 in cells treated with different PREP-modifications and for PREP-inhibition to decrease the colocalization.

4 MATERIAL AND METHODS

4.1 Cell culture and PREP-modifications

Human embryonic kidney cells 293 (HEK-293) and hPREP knockout cell line created from them by using CRISPR/Cas9-silencing were maintained in DMEM (#D6429, Sigma-Aldrich) with 10 % fetal bovine serum (FBS) and 1 % penicillin streptomycin at +37 °C. For experiments (Table 1), cells were grown on coverslips on 12-well plates and allowed to attach overnight before treatments. In inhibitor experiments, cells were exposed to either 10 µM DMSO, 1 µM KYP-2047 or 10 µM KYP-2047 for 4 hours. Incubation time was chosen based on previous experience with KYP-2047, to ensure results without compromising the viability of cells. In overexpression experiments, cells were transfected with either plasmid OTTC414 (hPREP) or OTTC446 (S554A hPREP). Lipofectamine 3000 reagent (#L3000015, Thermo Fisher) was first diluted with Opti-MEM (#31985070, Thermo Fisher) and was added 1:1 to mixture of 1 ug of plasmid-DNA, Opti-MEM and P3000 reagent to allow complex formation. Mixture was added to wells containing cells and incubated for 24 hours. No treatment was done for knockout cells.

Table 1.

Experiment	**Treatment**	**Cell line**
Inhibitor	Control (no treatment)	HEK-293
	10 µM DMSO	HEK-293
	1 µM KYP-2047	HEK-293
	10 µM KYP-2047	HEK-293
Overexpression	Control (no treatment)	HEK-293
	Lipofectamine control (LFC)	HEK-293
	OTTC414 (active hPREP)	HEK-293
	OTTC446 (inactive hPREP)	HEK-293
hPREP knockout	No treatment	hPREP knockout cell line from HEK-293

4.2 Immunofluorescence

After treatment, cells were fixed in 4 % paraformaldehyde for 10 minutes and then permeabilized with 0.5 % Triton X-100 in phosphate buffered saline (PBS) for 5 minutes. 10 % goat normal serum (Vector Laboratories) in 0.5 % Triton X-100 in PBS was used for blocking at room temperature for 30 minutes and 1 % goat normal serum was used for antibody dilutions. Coverslips were left in primary antibody anti-Bcl-2 (ab692, Abcam) 1:250 or anti-Beclin 1 (ab62557, Abcam) 1:500 overnight in room temperature. An Alexa Fluor 488® conjugated secondary antibody (ab150117, Abcam) 1:300 was used for Bcl-2 and a Texas Red® conjugated secondary antibody (#31506, Thermo Scientific) 1:500 was used for Beclin 1 for 2 hours in room temperature. All samples were stained first with anti-Bcl-2 and Alexa Fluor and with anti-Beclin 1 and Texas Red after that. After staining, coverslips were inverted to objective glass with Vectashield mounting media with DAPI (Vector Laboratories) and attached with nail polish. Samples were stored in dark and +4 °C until photographing.

4.3 Microscopy and analysis

A confocal microscope Leica TSC SP5 (HCX APO 63x/1,30 Corr CS 21 glycerol immersion objective) with Leica Application Suite X software was used for photographing samples. Cells

were imaged in z-stacks. At least 10 z-stacks containing a different individual cell were taken from each sample group for colocalization analysis. In addition, some close-up images were taken. Colocalization analysis was performed in Imaris (Bitplane, Switzerland) as described in Supplement I. Statistics and graphs were done with Prism (GraphPad Software, USA), with 95 % confidence interval using either unpaired t-test or one-way ANOVA and Tukey's post hoc test.

5 RESULTS

5.1 Confocal microscopy

Staining patterns of cells in inhibitor experiment were similar in all treatments (Figure 4). Staining of Beclin 1 was more intensive in the nucleus than in other parts of the cell. This staining pattern could be seen in all the samples from other experiments as well (Figure 5, Figure 6). In overexpression experiment (Figure 5), staining of Bcl-2 was more diffuse in cells transfected with inactive hPREP (OTTC446) compared to lipofectamine control (LFC) and active hPREP (OTTC414), where staining was more grain-like with larger and brighter spots visible, especially in OTTC414 samples. Staining of Beclin 1 was more intensive in nucleus than in other parts of the cell especially in OTTC446 samples where staining was very weak outside nucleus. In KO-cells (Figure 6), staining of Bcl-2 was also more visible in nucleus in addition to Beclin 1 compared to regular HEK-293-cells used as control.

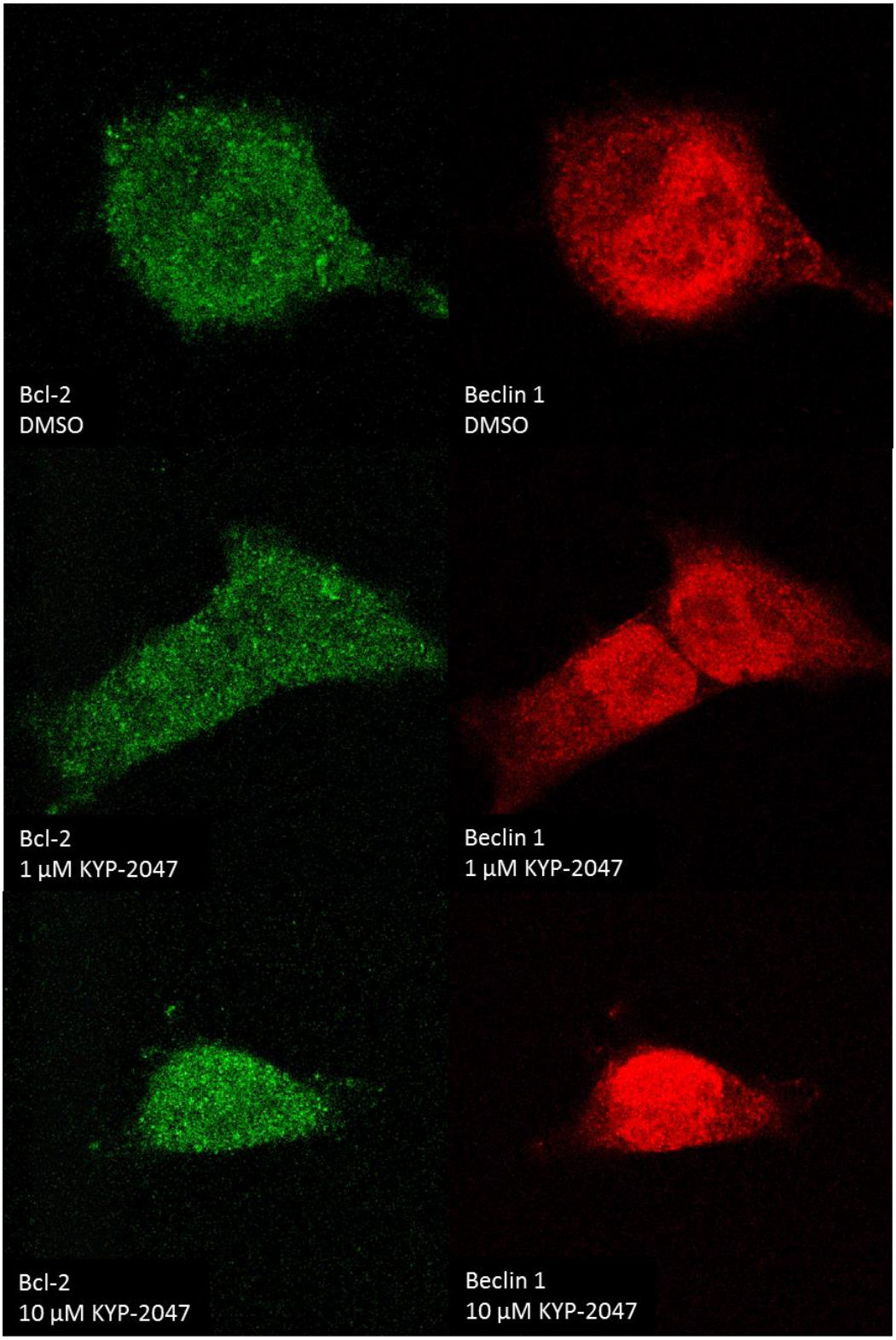

Figure 4. Confocal microscopy images from PREP-inhibition experiment showing staining of Bcl-2 and Beclin 1. 10 µM DMSO was used as control and PREP-inhibitor KYP-2047 was used in two different concentrations, 1 µM and 10 µM.

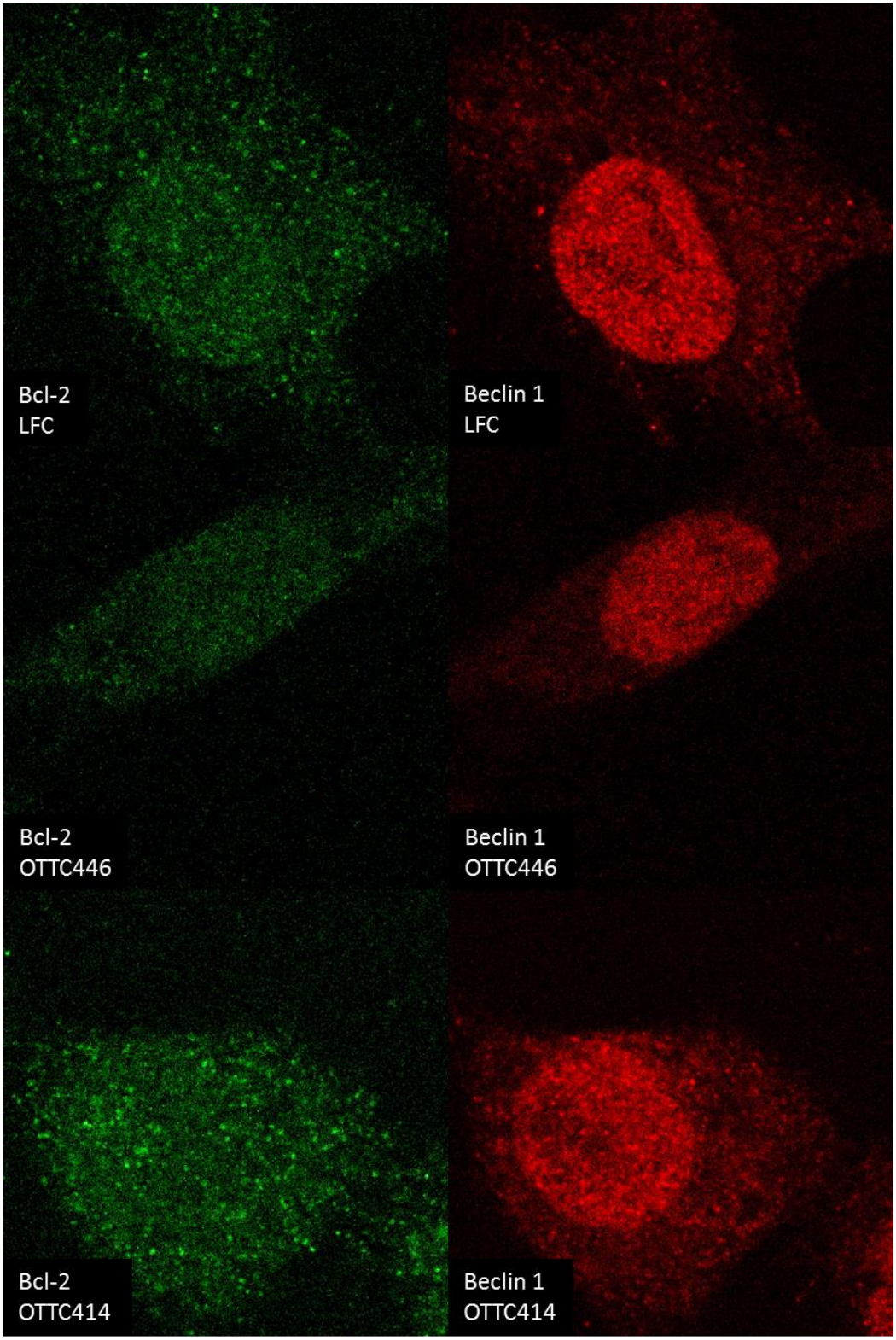

Figure 5. Confocal microscopy images from overexpression experiment showing staining of Bcl-2 and Beclin 1. Cells were transfected with either inactive hPREP plasmid (OTTC446) or active hPREP plasmid (OTTC414) and lipofectamine control (LFC) was used as control.

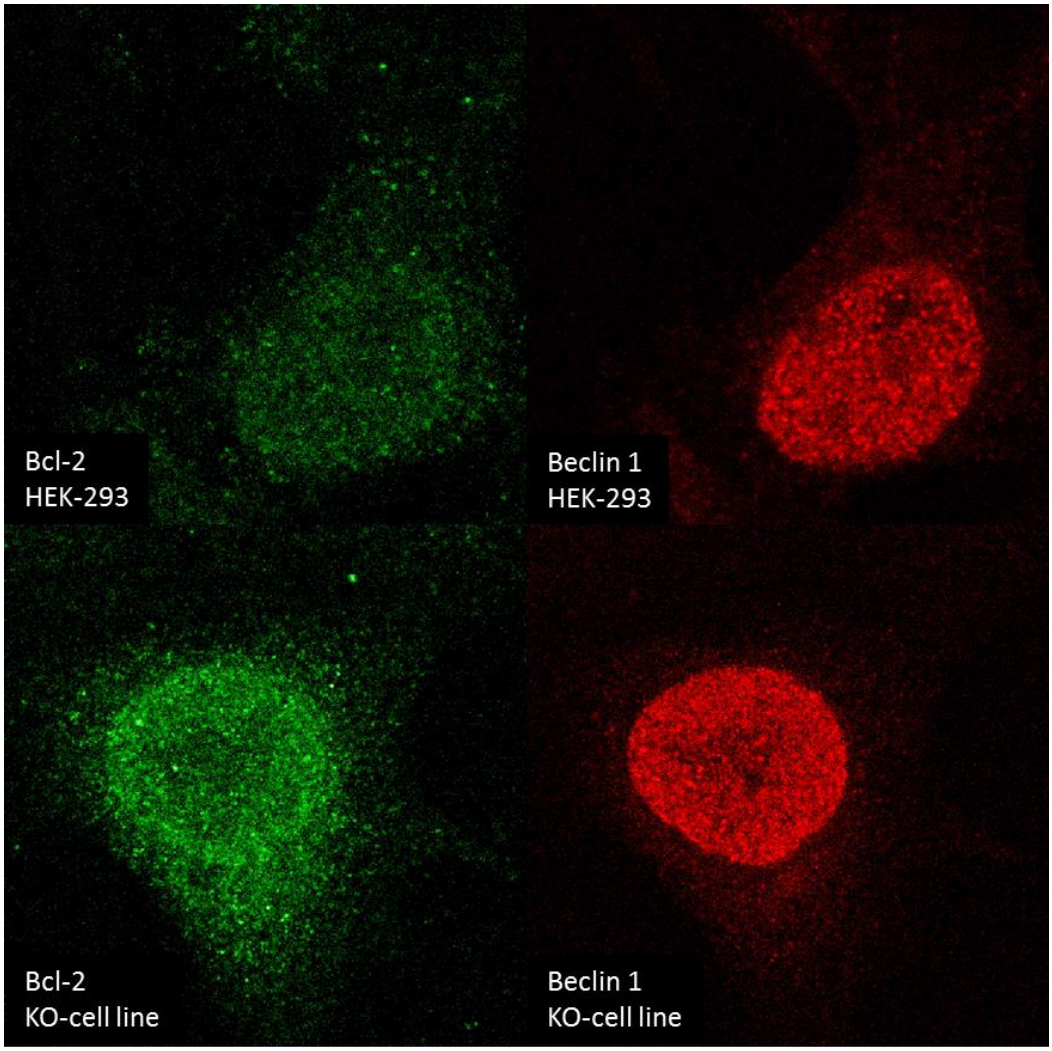

Figure 6. Confocal microscopy images from regular HEK-293 cells and KO-cell line created from them showing staining of Bcl-2 and Beclin 1.

5.2 Colocalization of Bcl-2 and Beclin 1

In inhibitor experiment, there were no significant differences in colocalization of Bcl-2 or Beclin 1 with one another (Figure 7). Colocalization for Bcl-2 with Beclin 1 was 97.99% for DMSO control, 98.76% for 1 µM KYP-2047 and 96.83% for 10 µM KYP-2047 ($F_{2,21}$=2.61, p=0.097, One-way ANOVA). Colocalization for Beclin 1 with Bcl-2 was 97.92% for DMSO control, 98.85% for 1 µM KYP-2047 and 98.45% for 10 µM KYP-2047 ($F_{2,21}$=0.45, p=0.64, One-way ANOVA).

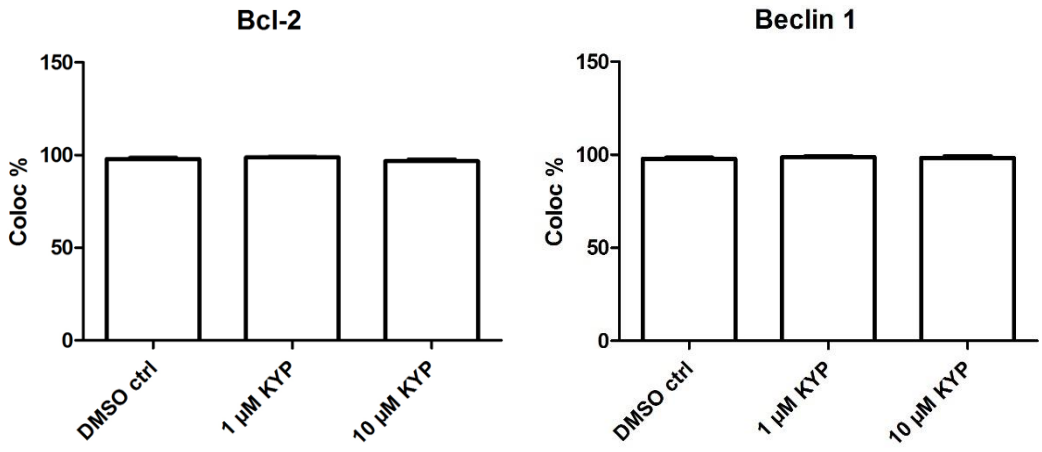

Figure 7. Colocalization of Bcl-2 with Beclin 1 and Beclin 1 with Bcl-2 in inhibitor experiment. There were no significant differences in colocalization between different treatments for Bcl-2 ($F_{2,21}$=2.61, p=0.097, One-way ANOVA) or Beclin 1 ($F_{2,21}$=0.45, p=0.64, One-way ANOVA).

There was a significant difference in colocalization of Bcl-2 with Beclin 1 between LFC control and OTTC446 samples in overexpression experiment (Figure 8). Colocalization for Bcl-2 was 97.30% for LFC control, 92.92% for OTTC446 and 94.77% for OTTC414 ($F_{2,21}$=5.10, p=0.0156, One-way ANOVA and Tukey's post hoc test). There were no significant differences in colocalization of Beclin 1 with Bcl-2 between the treatments in overexpression experiment. Colocalization for Beclin 1 was 98.40% for LFC control, 97.53% for OTTC446 and 97.77% for OTTC414 ($F_{2,21}$=0.35, p=0.71, One-way ANOVA).

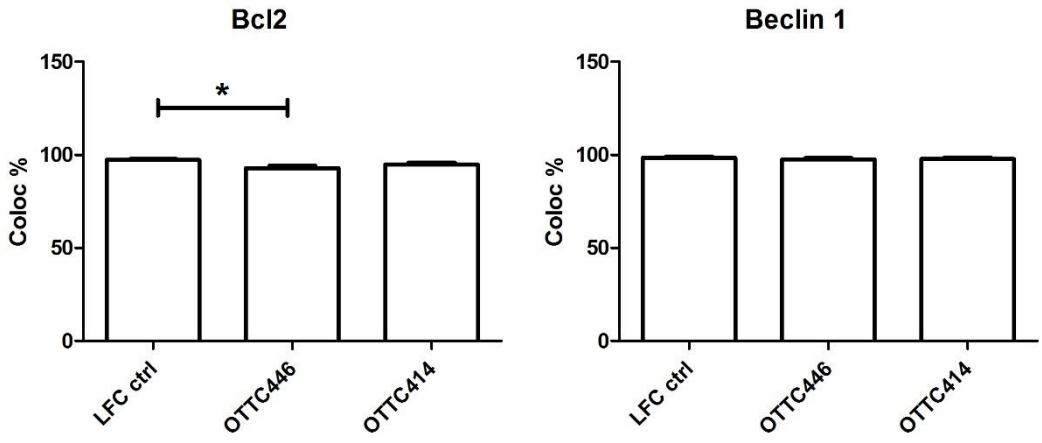

Figure 8. Colocalization of Bcl-2 with Beclin 1 and Beclin 1 with Bcl-2 in overexpression experiment. There was a significant difference in colocalization of Bcl-2 between lipofectamine control (LFC) and OTTC446 (inactive hPREP) ($F_{2,21}$=5.10, p=0.0156, One-way ANOVA and Tukey's post hoc test). There were no significant differences in colocalization between different treatments for Beclin 1 ($F_{2,21}$=0.35, p=0.71, One-way ANOVA).

There were no significant differences in colocalization of Bcl-2 or Beclin 1 with one another in KO-cell line compared to regular HEK-293 cells (Figure 9). Colocalization for Bcl-2 with Beclin 1 was 95.71% in HEK-293 cells and 96.69% in KO-cell line ($t_{1,13}$=0.69, p=0.50, Unpaired t-test). Colocalization for Beclin 1 with Bcl-2 was 99.60% in HEK-293 cells and 99.17% in KO-cell line ($t_{1,13}$=1.14, p=0.27, Unpaired t-test).

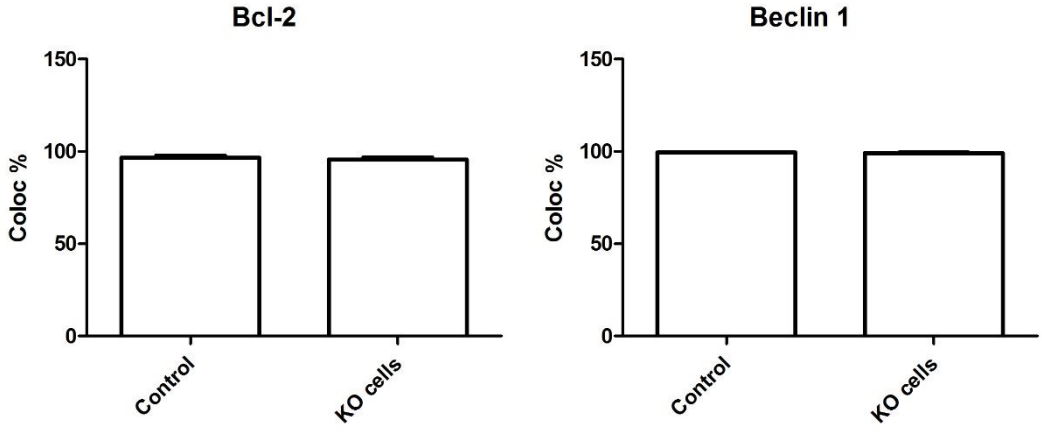

Figure 9. Colocalization of Bcl-2 with Beclin 1 and Beclin 1 with Bcl-2 in regular HEK-293-cells and KO-cell line. There were no significant differences in colocalization between the cell lines for Bcl-2 ($t_{1,13}$=0.69, p=0.50, Unpaired t-test) or Beclin 1 ($t_{1,13}$=1.14, p=0.27, Unpaired t-test).

5.3 Intensity of Beclin 1 in nuclei

Staining of Beclin 1 was more intensive in nucleus than in other parts of the cell as can be seen from the microscopy images. To see if there were any changes in the intensity, I decided to compare the intensities of Beclin 1 in nucleus between different treatments within each of the three experimental settings. There were no statistically significant differences in any of the experiments (Figure 10). In the inhibitor experiment, there was a small increase in the intensity in 1 µM KYP-2047 samples compared to control and 10 µM KYP-2047. Intensity of Beclin 1 was 35.21 for DMSO control, 44.79 for 1 µM KYP-2047 and 35.15 for 10 µM KYP-2047 ($F_{2,21}$=1.04, p=0.37, One-way ANOVA). In the overexpression experiment, Beclin 1 intensity was 19.25 for LFC control, 19.95 for OTTC446 and 20.28 for OTTC414 ($F_{2,21}$=0.054, p=0.95, One-way ANOVA). In the KO-cell line, the intensity of Beclin 1 was slightly decreased compared to regular HEK-293 cells. The intensity was 23.51 in HEK-293 cells and 17.58 in KO-cell line ($t_{1,13}$=1.55, p=0.14, Unpaired t-test).

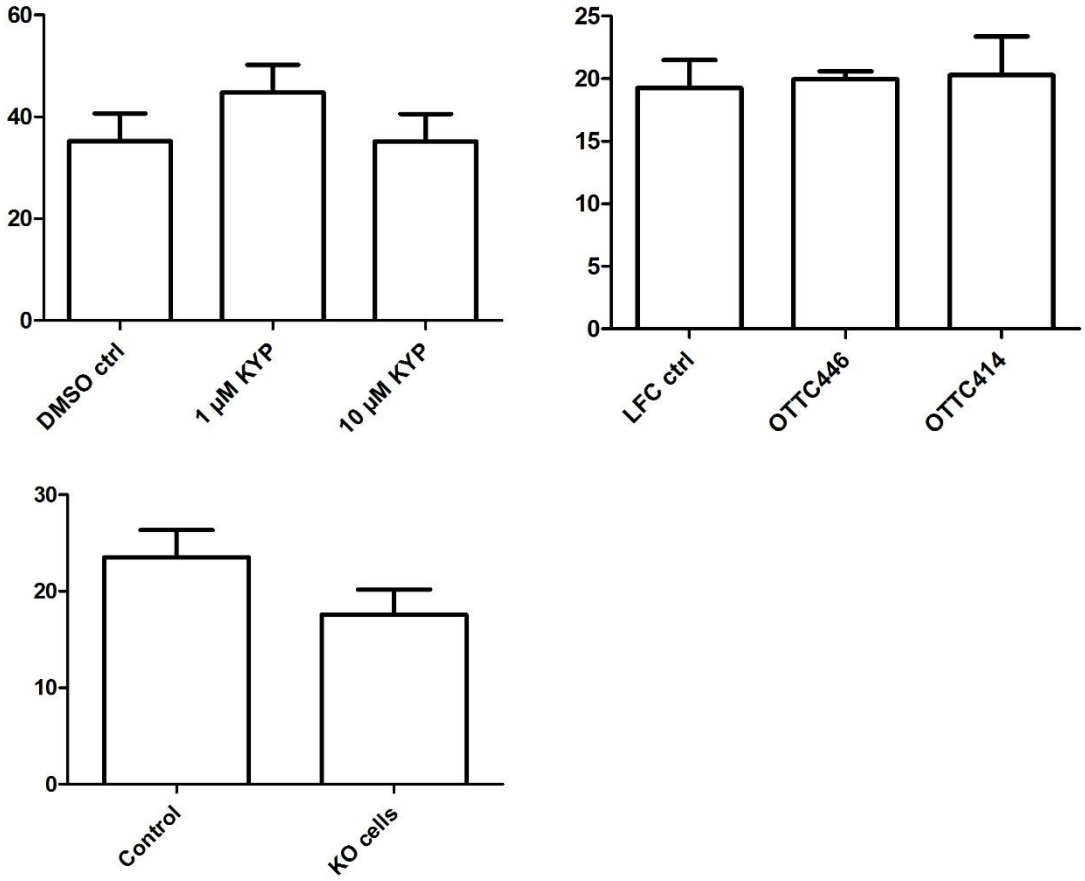

Figure 10. Intensity of Beclin 1 in nuclei. There were no significant differences in the intensity in any of the experiments: inhibitor experiment ($F_{2,21}$=1.04, p=0.37, One-way ANOVA), overexpression experiment ($F_{2,21}$=0.054, p=0.95, One-way ANOVA) or in KO-cell line ($t_{1,13}$=1.55, p=0.14, Unpaired t-test).

6 DISCUSSION

6.1 Microscopy and staining patterns

In the microscopy images (Figures 3-5), a staining pattern of stronger intensity of Beclin 1 in nuclei was seen in all the samples. Endogenous Beclin 1 localizes to TGN (Kihara et al 2001), but colocalization of Beclin 1 with mitochondria and ER has also been detected (Pattingre et al 2005). It is possible that there are some differences in localization of Beclin 1 depending on the cell line, since different cell lines were used in the studies, although Kihara et al (2001) used three different cell lines that gave similar results. Localization of Beclin 1 has not been studied in HEK-293 cells before.

Beclin 1 is essential in autophagosome formation which takes place in ER (Xie and Klionsky 2007). However, it has also tasks in nucleus independent of its function in autophagy, such as a role in mitotic chromosome congression (Fremont et al 2013) and repair of DNA damage (Xu et al 2017). In the current study, the intensity of Beclin 1 in the nuclei was stronger than in other parts of the cell which could indicate a stronger activity of its nuclear tasks compared to autophagy. One reason behind this staining pattern was the antibody used for immunofluorescence. The result could not be repeated with another anti-Beclin 1 antibody from the same manufacturer (ab207612, Abcam). This other antibody produced a staining pattern comparable to Bcl-2 staining showed in Figures 3-5, with a similar intensity in all parts of the cell. In western blot analysis (data not shown), both Beclin 1 antibodies showed one band of the same size, so it is unclear why they did not work in the same way in immunofluorescence staining. Most likely there was some small, undetectable difference between the two antibodies that lead to a different staining pattern in the cells.

Bcl-2 localizes on outer ER and mitochondrial membranes, but not to TGN as Beclin 1 does (Germain and Shore 2003). The staining pattern of Bcl-2 in Figures 3-5 was in line with the data from previous studies, although the exact location in cell could not be determined from the images, since only nuclear marker was used in staining (DAPI in mounting media). Bcl-2 seemed to be concentrated in certain areas of the cell and produce different staining patterns in different treatments. In samples with active PREP plasmid (OTTC414), the staining pattern was grainy compared to the more diffuse staining in samples with inactive PREP plasmid (OTTC446). Increased amount of PREP seemed to affect distribution of Bcl-2, since in the control samples the staining was more concentrated and grainy, although not with as many and as bright spots as in OTTC414, compared to inactive OTTC446.

One contributor to the detected staining patterns could be the microtubule network in the cells. Microtubule network is most likely important in autophagosome formation and trafficking and could also be involved in autophagosome maturation (Weidberg et al 2011). Tubulin modifications are also important contributors to regulation of autophagy (Geeraert et al 2010). Tubulin acetylation allows activation of JNK, which can then phosphorylate Bcl-2 and break the interaction with Beclin 1. Localization of Bcl-2 and Beclin 1 near microtubuli would thus be convenient.

6.2 Colocalization of Bcl-2 and Beclin 1

Relationship between Bcl-2 and Beclin 1 and its importance in regulation of autophagy is well established in the literature (Pattingre et al 2005). Their separation, caused by phosphorylation of either one, leads to induction of autophagy (Yang and Klionsky 2010). PREP seems to reduce formation of autophagosomes and its inhibition increases expression of Beclin 1, leading to enhanced autophagy (Savolainen et al 2014). Based on this previous knowledge, it was expected to see differences in colocalization of Bcl-2 and Beclin 1 in cells treated with different PREP-modifications. However, there were no significant differences in colocalization of Beclin 1 and Bcl-2 in any of the experiments but it was nearly 100 percent in all treatments. Colocalization results could of course be changed by changing parameters of the analysis (Supplement I), particularly amount, size and distance of Bcl-2 and Beclin 1 spots. Spots were created by visual estimate based on the intensity of the signal and by changing this threshold, the amount of Bcl-2 and Beclin 1 in cells could be altered. However, it is not likely that this would change the results that much but could compromise the reliability. The immunofluorescence stainings were performed successively to minimize the risk for cross-staining and there were no problems that could have affected the results. The antibodies used were chosen carefully. To improve the reliability of the results, a positive control should also be tested. In this case that would be a PREP-inducer, as now only inhibitors and different plasmids were tested. It would also be interesting to do immunofluorescence staining with antibodies for phosphorylated Bcl-2 and Beclin 1, but there are no reliable antibodies available for this purpose. The experiments could also be repeated in another cell line to confirm the results and make sure they are not specific to HEK-293 cell line.

Next step of the study would be to determine changes in autophagy in different treatments and to see if there are any differences. Autophagy is a complex process with several different steps, so multiple assays to detect autophagy should be chosen to get reliable results. Possible assay for measuring autophagy would be to study autophagy markers like LC3-II. It is used to detect autophagosome formation and its protein levels correlate with number of autophagosomes (Lumkwana et al 2017). Autophagy marker assay could be combined with assay to measure autophagic flux, which means the degradation rate of material through autophagy pathways (Lumkwana et al 2017). This is important, since the number of autophagosomes does not

directly indicate functional autophagy and its efficiency. There are several methods for measuring autophagic flux including fluorescence microscopy techniques (Loos et al 2014).

Colocalization of Bcl-2 and Beclin 1 was close to 100 percent in all treatments. Since rate of autophagy in cells was not detected, it is impossible to determine, if there were changes in autophagy that were not reflected as changes in colocalization of these two proteins. Interaction between Bcl-2 and Beclin 1 involves a BH3-domain located within Beclin 1 and there could be some differences in binding specificity or subpopulations of Bcl-2 or Beclin 1 that have different binding affinities (Maiuri et al 2007). If the interaction of Bcl-2 and Beclin 1 was interrupted only in certain subpopulations, it is possible it would not be detectable in the analysis and experiments performed in this study. Pattingre et al (2005) showed that only ER-targeted Bcl-2 inhibits autophagy, which supports the hypothesis of different functions and affinities for different subpopulations. Both Bcl-2 and Beclin 1 also have functions not related to autophagy, most important being apoptosis, main route for programmed cell death (Rubinstein and Kimchi 2012). Some of the cells seemed to be in bad condition, especially the nuclei, although none of the treatments should cause actual cell death. Especially the KO-cell line grew slowly and seemed poor in health. It is not likely that the cells were apoptotic, but it is important keep in mind, that the signalling pathways of autophagy and apoptosis are connected through multiple proteins and apoptosis can be regulated via autophagy and vice versa. There is usually a high level of autophagy in dying cells (Levine and Yuan 2005). Possible explanations for this include requirement for massive cell elimination on certain situations and hypothesis that autophagy is a self-clearance mechanism in dying cells. However, based on current evidence, autophagy seems to be above all a pro-survival mechanism in cells.

7 CONCLUSIONS

Autophagy is an essential pathway for cells to degrade intracellular components that are no longer needed or that can be harmful for the cells. It is important in cell survival and homeostasis and is connected to other pathways in cells such as apoptosis. Autophagy is related to several diseases such as cancer and neurodegenerative diseases. This has raised the interest to study autophagy in recent years. One of the molecules under investigation is PREP. PREP-inhibitor KYP-2047 has been shown to enhance clearance of α-synuclein protein aggregates by increased rate

of autophagy and by reducing dimerization (Savolainen et al 2014, Savolainen et al 2015). It also enhances expression of Beclin 1 in cells. Bcl-2 and Beclin 1 are important in mTOR-independent autophagy, as interference with their interaction increases the rate of autophagy. Although no differences in colocalization of Bcl-2 and Beclin 1 were found in this study where cells were treated with different PREP-modifications, it was found out that the colocalization is on high level in the cells. It is possible that even a small change in colocalization can affect the rate of autophagy or there might be subpopulations where the interaction is interrupted and these changes are so small that they are not detectable with the methods used in this experiment. Since autophagy is a complex process with several unclear aspects, it is important to study the pathways to discover possible cures for conditions where autophagy is compromised.

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SUPPLEMENT I

Colocalization analysis with Imaris

Colocalization analysis of Bcl-2 and Beclin 1 was done with Imaris software version 8.4.4. (Bitplane, Switzerland). First, surface was created for nucleus (cyan channel) with surface detail of 0,5 µm. Then, green (Bcl-2) and red (Beclin 1) channels were masked setting the voxels inside surface to 0. Spots were created for masked green and red channels using xy-size 0,4 µm (z=0,8 µm). Colocalization analysis for the spots was done with MathLab extension in Imaris with threshold of 0,3 µm.

Beclin 1 intensity in nucleus was measured by masking the red channel with voxels outside the surface as 0. Intensity mean and standard deviation for the masked channel were then obtained from surface properties.